Summary

The purpose of this study was to perform an independent laboratory validation of a method (PTRL Europe Study No.: P 1578 G) for the determination of pethoxamid drinking water.

This method is referenced as AGR/MOA/PTX-9 at Eurofins Agroscience Services Chem SAS. The method involves the extraction of the samples using solid phase extraction (SPE) using C18 cartridges with elution of the analyte using acetonitrile. An aliquot was diluted prior to quantification by LC-MS/MS. The limit of quantification (LOQ) is 0.1 µg/L.

The method was validated in compliance with European guidelines for residue analytical methods SANCO/825/00 rev.8.1 (16/11/2010). A full validation set was performed to demonstrate that the method allows accurate determination of pethoxamid residues in drinking water matrix.

For method validation, after fortification with the analyte, the following specimens were analysed by LC-MS/MS:

- 5 specimens fortified at LOQ level: 0.1 µg/L,
- 5 specimens fortified at 1 µg/L (10 × LOQ),
- 2 unfortified specimens,
- 1 reagent blank.

1. Introduction

The purpose of this study was to perform an independent laboratory validation of a method (PTRL Europe Study No.: P 1578 G) for the determination of pethoxamid drinking water.

The limit of quantification (LOQ) was 0.1 µg/L.

2. Analytical method for pethoxamid

2.1. Reference of the method

The method used during this study is referenced at Eurofins Agroscience Services Chem SAS under the number AGR/MOA/PTX-9.

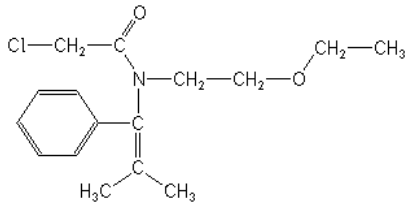
2.2. Principle of method AGR/MOA/PTX-9

This analytical method involved the extraction of the samples using solid phase extraction (SPE) using C18 cartridges with elution of the analyte using acetonitrile. An aliquot was thereof diluted prior to quantification by LC-MS/MS. The limit of quantification (LOQ) is 0.1 µg/L.

The residues of pethoxamid were analysed by LC-MS/MS using two transitions.

2.3. Reference items

Common name:	Pethoxamid
Chemical name (IUPAC):	2-chloro-*N*-(2-ethoxyethyl)-*N*-(2-methyl-1-phenylprop-1-enyl)acetamide
CAS-Registry-No.:	[106700-29-2]
Molecular formula:	$C_{16}H_{22}ClNO_2$
Molecular mass:	295.8 g/mol
Supplier:	Cheminova
Batch:	P1351-BKA-89
Purity:	99.8%
Storage condition:	Temperature set at -20°C
Expiry date:	12 Nov 2014



The certificate of analysis is located in appendix 1. The reference item was stored at a nominal temperature -20°C whereas the certificate of analysis indicates a temperature <-20°C. This was considered to have no impact on the study. The sponsor confirms that <-20°C means frozen state and not deep frozen state.

2.4. Test system

The validation was carried out on drinking water, obtained from Vergèze (30), France. Upon receipt, the water was stored at a temperature set at 4 °C.

The water was characterized by Eurofins Institut Pasteur Lille (a non-GLP facility, however, COFRAC certified), 778 rue de la Croix Verte, Parc Euromedecine, 34000 Montpellier, France.

Details of the characterization results are as follows:

Specimen	pH (15.2°C)	Total Hardness (°F)	Total Suspended Solids (mg/L)	Calcium (mg/L)	Magnesium (mg/L)	Dissolved Organic Carbon (mg/L)
Method	NF T 90-008	By calculation	NF EN 872	NF EN ISO 14911	NF EN ISO 14911	NF EN 1484
Drinking Water N°291	6.9	8.0	<2	24	4.8	<0.4

2.5. Detailed description of method AGR/MOA/PTX-9

2.5.1. Preparation and use of the standard solutions

2.5.1.1. Pethoxamid stock solution

- Between 2 and 50 mg of pethoxamid are accurately weighed into a brown flask.
- Adequate volume of acetone for pethoxamid is added in order to obtain stock solution at 1000 µg/mL, taking into account the chemical purity. This solution is sonicated until total dissolution.

The standard solutions of pethoxamid were stored at a temperature set at 4°C. The standard solution of pethoxamid was proven to be stable for 10 days.

2.5.1.2. Pethoxamid fortification solutions

For fortifications, appropriate dilutions of the pethoxamid primary stock solution were performed in acetone to obtain solutions at 0.1, 1 and 10 µg/mL.

These solutions were freshly prepared.

2.5.1.3. Pethoxamid calibration solutions

Appropriate serial dilutions of the stock solution were performed in acetonitrile/ultra-pure water (50/50, v/v) at 10 µg/mL. Appropriate serial dilutions of this solution were performed in acetonitrile/ultra-pure water (50/50, v/v) to obtain solutions at:

0.0005 - 0.001 – 0.0025 – 0.005 – 0.01 – 0.025 – 0.05 – 0.1 and 0.25 µg/mL.

The calibration solutions were freshly prepared.

Preparation of calibration standards

Standards mentioned above were 10-fold diluted in acetonitrile/ultra-pure water (50/50, v/v).

The following calibration solutions were prepared:

0.05 – 0.1 – 0.25 – 0.5 – 1 – 2.5 – 5 – 10 and 25 ng/mL.

2.5.2. Analytical supplies and apparatus

2.5.2.1. Apparatus

- HPLC pump (Shimadzu LC20AD)
- HPLC injector (CTC Analytics HTC Pal)
- HPLC oven (Shimadzu CTO-20AC)
- LC-MS/MS: API 4000 (Sciex)
- HPLC column Thermo Aquasil (C18), 150 x 3 mmm i.d., 3 µm particle size (supplier, ref.)
- HPLC pre-column Thermo Hypersil Gold, 10 x 3 mmm i.d., 5 µm particle size (Fischer, ref. 10544085)
- Cartridge SPE BondElutC18 (500 mg/6mL) (Agilent, ref.12102052)
- Polypropylene centrifugation tubes
- Precision balance (Mettler)
- Standard laboratory glassware (volumetric flasks, measuring cylinders)
- Ultrasonic bath (Bioblock)
- Various pipettes (Thermo Scientific)

2.5.2.2. Reagents and chemical compounds used

All solvents were HPLC-grade.

- Acetic acid (VWR, ref. 100063.1000)
- Acetone (Sigma ref 34850-2.5l)
- Acetonitrile (VWR, ref. 83640.320)
- Ammonium acetate (Fisher ref. A/3440/50)
- Ultra-pure water (Eurofins Agroscience Services Chem SAS)

2.5.3. Sample Extraction

Sample Fortification

- Pipette (100 mL) of water sample into a polypropylene flask (250 mL size). Sample fortification, if required, was to be carried out at this point. At least one untreated control and two control samples fortified with a known amount of pethoxamid should be analysed alongside each batch of samples to demonstrate acceptable performance of the method.

Matrix (mL)	Fortification level (µg/L)	Volume to use (mL)	Solution to use (µg/mL)
Drinking water (100)	0.1	0.1	0.1
Drinking water (100)	1	0.1	1.0

Solid Phase Extraction

- The Varian C18 SPE cartridges (500mg, 6 mL) were placed on SPE station(s) equipped with Teflon stop cocks, vacuum manifold(s) attached to water suction pump(s) or vacuum pumps.
- 75-mL SPE reservoirs with adapters were fitted onto cartridges.
- SPE cartridges were pre-conditioned with 3 mL of acetonitrile and 3 mL of ultra-pure water.
- The 100-mL water specimens were added into the reservoirs and the water drawn through the cartridge drop wise, the extracted water discarded.
- The SPE cartridges were washed with 20 mL of de-ionized water, followed by 3 mL acetonitrile/ultra-pure water (50/50,v/v)
- 2.5 mL of acetonitrile was added to the cartridge and drawn through the cartridge to elute the analyte from the C18 material.
- The acetonitrile eluate was collected.
- A 0.1 mL aliquot was diluted with acetonitrile/ultra-pure water (50/50, v/v) to a final volume of 1.0 mL ready for final determination by LC-MS/MS.

2.5.4. Parameters for chromatographic analysis

2.5.4.1. Operating conditions

The following parameters were used during the study.

LC-MS/MS:

- Pump + Autosampler: LC20AD, Shimadzu + HTC Pal, CTC Analytics
- Oven: CTO-20AC, Shimadzu
- Detector: API 4000 (Sciex)
- Data Acquisition: Analyst 1.5.1, Sciex
- Column HPLC: Thermo Aquasil (C_{18}), 150 x 3 mmm i.d., 3 µm particle size
- Pre column HPLC: Thermo Hypersil Gold, 10 x 3 mmm i.d., 5 µm particle size
- Column temperature: 35 °C
- Retention time: approximately 7.23 minutes
- Injection volume: 10 µL (depending on sensitivity)
- Flow: 0.25 mL/minute
- Mobile phase: Solvent A: 0.1 % acetic acid and 0.01 M ammonium acetate in ultra-pure water/acetonitrile (80/20, v/v)
  Solvent B: 0.1 % acetic acid and 0.01 M ammonium acetate in acetonitrile/ultra-pure water (80/20, v/v)
- Gradient:

Time (minute)	% A	% B
0.00	30	70
3.00	0	100
9.00	0	100
9.10	30	70
12.00	30	70

- Ionisation mode: ESI^+
- Scan Type: MRM
- Gas supply: Nitrogen

Analyte	Parent ion (*m/z*)	Daughter ion (*m/z*)	DP (V)	EP (V)	CXP (V)	CE (V)	Dwell (ms)
Pethoxamid	296.1	131.1 (primary)	36	10	10	30	500
	296.1	250.1 (confirmatory)	36	10	22	15	500

DP : declustering potential CE : collision energy CXP : collision cell exit potential EP: entrance potential

CAD (collision gas)	4	**TEM (°C)**	450
CUR (curtain gas)	12	**RESOLUTION Q1**	Unit
GS1 (ion source gas 1)	50	**RESOLUTION Q3**	Unit
GS2 (ion source gas 2)	60		
IS (ion spray voltage)	4500		

Integration and calibration parameters

Response	Area	**Type of regression**	Linear
Type of response	External	**Weighting**	1 / X
Unit	ng/mL	**Intercept**	No

2.5.4.2. Calibration

Quantitative determination was carried out by external standardisation. A calibration curve was injected prior to analysis of the sample list. Besides, at least one quality control was injected every four injections to check the absence of signal deviation.

The determination coefficient R^2 was found to be ≥ 0.990.

Typical calibration curves and chromatograms for LC-MS/MS are presented in Appendix 2.

2.5.4.3. Result calculation

The chromatographic system was calibrated using a calibration curve of pethoxamid standards.

A linear calibration curve was calculated using the method of least squares (1/x weighting):

$$Y = A \times C + B$$

Y = detector response (as peak area)

A = slope of the linear least squares fit of the calibration curve

C = Concentration determined from standard curve (ng/mL)

B = Y-intercept of the linear least squares fit of the calibration curve

The concentration determined from standard curve is $C = \frac{(Y - B)}{A}$

The residue of pethoxamid in each test specimen is calculated as follows:

$$\text{Residue}\ (\mu g / L) = \frac{V_1 \times V_f}{M \times V_2} \times extract\ \ concentration\ (\text{ng} / mL)$$

Where:

V_1	=	extraction volume (2.5 mL)
V_2	=	aliquot volume (0.1 mL)
V_f	=	final volume (1 mL)
M	=	sample volume (100 mL)
Extract concentration	=	calculated concentration in final extract

Procedural recovery data from fortified samples are calculated via the following equation:

$$\text{Recovery}\ (\%) = \frac{A}{S} \times 100$$

Where:

A = concentration of pethoxamid found in spiked sample (µg/L).

S = concentration of pethoxamid added in spiked sample (µg/L).